REMARKS

Amendments

Claims 1-28, 33-35, 37, 40, 41, 44 and 45 have been canceled, and claims 29, 42 and 43 have been amended. Upon entry of the amendment, claims 29-32, 36,38, 39, 42, 43 and 46 will be pending. Support for the amendment can be found in the specification, for example, in Figure 2B, and in the claims as originally filed.

Rejections

Rejections under 35 U.S.C. § 101

The Examiner has rejected claims 29-32, 36, 38-40 and 42-46 because the claimed invention is allegedly not supported by either a specific or substantial asserted utility or a well-established utility for the reasons of record.

Applicant does not agree. Amended claim 1 is drawn to a transgenic mouse whose genome comprises a disruption in the endogenous CX2 gene, wherein said disruption comprises replacement of nucleotides corresponding to bases 327 through 422 of SEQ ID NO:1 with a LacZ-Neo cassette.

1. The Utility Requirement

Section 101 of the Patent Act of 1952, 35 U.S.C. § 101, provides that "whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof," may obtain a patent on the invention or discovery.

According to the Federal Circuit:

> The threshold of utility is not high: An invention is "useful" under section 101 if it is capable of providing some identifiable benefit. See Brenner v. Manson, 383 U.S. 519, 534, 16 L. Ed. 2d 69, 86 S. Ct. 1033 (1966); Brooktree Corp. v. Advanced Micro Devices, Inc., 977 F.2d 1555, 1571 (Fed. Cir. 1992) ("To violate § 101 the claimed device must be totally incapable of achieving a useful result"); Fuller v. Berger, 120 F. 274, 275 (7th Cir. 1903) (test for utility is whether invention "is incapable of serving any beneficial end").

(*Juicy Whip v Orange Bang*, 185 F.3d 1364; 51 U.S.P.Q.2d 1700 (Fed. Cir. 1999)(emphasis added)).

2. Well-Established Utility

According to 35 U.S.C. § 101, "[w]hoever invents . . . any new and useful . . . composition of matter may obtain a patent therefore. . . . "

Under the Patent Office's Utility Requirement Guidelines:

> If at any time during the examination, it becomes readily apparent that the claimed invention has a well-established utility, do not impose a rejection based on lack of utility. An invention has a well-established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., properties or applications of a product or process), and (ii) the utility is specific, substantial, and credible.

Applicant submits that in light of arguments of record, a person of ordinary skill in the art would immediately appreciate why the invention is useful. Thus, it cannot be reasonably debated that a person of ordinary skill in the art would not immediately appreciate why the invention is useful: for determining gene function.

3. Substantial Utility

The Examiner argues that the asserted utilities are not substantial.

According to the MPEP:

> A "substantial utility" defines a "real world" use. Utilities that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use are not substantial utilities. . . . the following are examples of situations that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use and, therefore, do not define "substantial utilities":
>
> (A) Basic research such as studying the properties of the claimed product itself or the mechanisms in which the material is involved;
>
> Office personnel must be careful not to interpret the phrase "immediate benefit to the public" or similar formulations in other cases to mean that products or services based on the claimed invention must be "currently available" to the public in order to satisfy the utility requirement. See, e.g., Brenner v. Manson, 383 U.S. 519, 534-35, 148 USPQ 689, 695 (1966). Rather, any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a "substantial" utility.

(MPEP § 2107.01 I)(emphasis added).

The MPEP additionally provides:

> Office personnel must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm. Labels such as "research tool," "intermediate" or "for research purposes" are not helpful in determining if an applicant has identified a specific and substantial utility for the invention.

(MPEP § 2107.01, I).

A use is not substantial where further research is required to identify any use. This is not the case in the present application. Knockout mice have a well-known use in the study of gene function. In the present case, the instant invention does not require further research to establish a utility. Applicant has determined that the CX2 gene is associated with, for example, seizures and metabolism. No further research is required to establish any use. The invention has a "real world use" – as demonstrated by the delivery of the claimed invention to at least one large pharmaceutical company. Whether additional research is required to identify therapeutic agents targeting the CX2 gene or to further characterize the function of the CX2 gene is irrelevant to whether the claimed invention has satisfied the utility requirement (see, for example, *In re Brana*, "Usefulness in patent laws . . . necessarily includes the expectation of further research and development.")

With regard to the commercial sale, Applicant does not agree with the Examiner's definition of commercial success. Commercial success must be defined in the context of the relevant market. In the relevant market of pharmaceutical drug development, the sale of single mouse model to a large pharmaceutical company amounts to recognition of that mouse as a research tool for drug development. Moreover, it is not the dollar value attached to a product, but rather the fact that actual use of the mouse by a pharmaceutical company logically satisfies the practical utility requirement of section 101. It cannot be reasonably argued that a claimed invention which is actually being used for the same purpose asserted in the specification, lacks patentable utility. According to the guidelines, if one skilled in the art would immediately recognize the use of a claimed invention (and such use is credible, specific and substantial) then the utility requirement is met. In the present case, this recognition is clearly demonstrated by the delivery of the invention to a large pharmaceutical company.

With regard to substantiality, according to the MPEP:

> **any reasonable use** that an applicant has identified for the invention that can be viewed as providing a public benefit **should be accepted as sufficient,** at least with regard to defining a "substantial" utility.

Certainly providing an *in vivo* model for studying the function of the CX2 gene is a reasonable use.

In addition, the MPEP specifically cautions Examiners not to get confused by labeling inventions as research tools:

> Office personnel must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm. Labels such as "research tool," "intermediate" or "for research purposes" are not helpful in determining if an applicant has identified a specific and substantial utility for the invention.

Applicant respectfully submits that the Examiner has done what the MPEP specifically cautions against, by providing: "[a]n assessment that focuses on whether an invention is useful only in a research setting thus does not address whether the invention is in fact "useful" in a patent sense."

4. Specific Utility

The Examiner states that the asserted uses are not specific.

According to the MPEP, "specific utility" means "specific" to the subject matter claimed as compared to a "general utility" that would be applicable to the broad class of the invention (MPEP 2107.01). Use of the CX2 -/- and +/- mice to study the function of the CX2 gene and the association of the CX2 gene with, for example, metabolism or seizures, is specific to this mouse. Even if there were many other genes associated with these phenotypes, only the CX2 knockout mouse (as opposed to all other knockout mice) would be used to study the specific role of this gene. The Examiner is respectfully requested to explain (1) how the asserted utility of determining the function of the CX2 gene would be applicable to all other knockout mice; and (2) how the asserted use of studying the association of the CX2 gene with seizures and metabolism would be applicable to all other knockout mice. The Examiner is requested to explain how all other knockout mice would be used to study the function of the CX2 gene.

In addition, the mice within the scope of claim 29 contain a *lacZ* gene. Their use in studying gene expression is clearly recognized by those skilled in the art:

> **Null-reporter alleles should be created**
> The project should generate alleles that are as uniform as possible, to allow efficient production and comparison of mouse phenotypes. The alleles should achieve a balance of utility, flexibility, throughput and cost. A null allele is an indispensable starting point for studying the function of every gene. Inserting a reporter gene (e.g., P-galactosidase or green fluorescent protein) allows a rapid assessment of which cell types normally support the expression of that gene.

(Austin et al., Nature Genetics (2004) 36(9):921-24, 922)(emphasis in original; emphasis added)(copy attached). As is well understood in the art, the *lacZ* gene is inserted into the endogenous gene. In this case, the *lacZ* gene was inserted into the locus of the CX2 gene. Expression is driven by the endogenous promoter. Expression of the *lacZ* gene indicates where the gene is expressed. This use is specific for this mouse – knockout mice in general cannot be used for this purpose. The Examiner is respectfully requested to explain how all other knockout mice would be used to study expression of the CX2 gene.

5. In re Brana

Applicant submits that the legal principles as well as the facts of *Brana* are applicable to the present case. In *Brana*, the Board held that the applicant's specification failed to disclose a specific disease against which the claimed compounds were useful. The Federal Circuit reversed and held that the mouse tumor model represented a specific disease against which the compounds were effective. It is Applicant's position that a mouse demonstrating, for example, increased glucose tolerance, is sufficient to establish the animal's use as a model for metabolic disorders and diseases. As in *Brana*, confirmation of the phenotype in humans is unnecessary. In *Brana*, the PTO was aware of the asserted use against the mouse tumor lines but did not find the use specific – as in the present case:

> Applicants' specification, however, also states that the claimed compounds have "a better action and a better action spectrum as antitumor substances" than known compounds, specifically those analyzed in Paull. As previously noted, see supra note 4, Paull grouped various benzo [de]isoquinoline-1,3-diones, which had previously been tested in vivo for antitumor activity against two lymphocytic leukemia tumor models (P388 and L1210), into various structural classifications and analyzed the test results of the groups (i.e. what percent of the compounds in the particular group showed success against the tumor models). Since one of the tested compounds, NSC 308847, was found

to be highly effective against these two lymphocytic leukemia tumor models, 14 applicants' favorable comparison implicitly asserts that their claimed compounds are highly effective (i.e. useful) against lymphocytic leukemia. An alleged use against this particular type of cancer is much more specific than the vaguely intimated uses rejected by the courts in Kirk and Kawai. See, e.g., Cross v. Iizuka, 753 F.2d at 1048, 224 USPQ at 745 (finding the disclosed practical utility for the claimed compounds -- the inhibition of thromboxane synthetase in human or bovine platelet microsomes -- sufficiently specific to satisfy the threshold requirement in Kirk and Kawai.)

The Commissioner contends, however, that P388 and L1210 are not diseases since the only way an animal can get sick from P388 is by a direct injection of the cell line. The Commissioner therefore concludes that applicants' reference to Paull in their specification does not provide a specific disease against which the claimed compounds can be used. We disagree.

(*Brana* at 1440). The court went on:

The ultimate issue is whether the Board correctly applied the Section 112 Para.1 enablement mandate and its implicit requirement of practical utility, or perhaps more accurately the underlying requirement of Section 101, to the facts of this case. As we have explained, the issue breaks down into two subsidiary issues: (1) whether a person of ordinary skill in the art would conclude that the applicants had sufficiently described particular diseases addressed by the invention, and (2) whether the Patent Act supports a requirement that makes human testing a prerequisite to patentability under the circumstances of this case.

The first subsidiary issue, whether the application adequately described particular diseases, calls for a judgment about what the various representations and discussions contained in the patent application's specification would say to a person of ordinary skill in the art. We have considered that question carefully, and, for the reasons we explained above in some detail, we conclude that the Board's judgment on this question was erroneous. Our conclusion rests on our understanding of what a person skilled in the art would gather from the various art cited, and from the statements in the application itself. We consider the Board's error to be sufficiently clear that it is reversible whether viewed as clear error or as resulting in an arbitrary and capricious decision.

The second subsidiary issue, whether human testing is a prerequisite to patentability, is a pure question of law: what does the practical utility requirement mean in a case of this kind. Under either our traditional standard or under the APA standard no deference is owed the Agency on a question of law, and none was accorded.

If the question concerning the standard of review, raised by the Commissioner, is to be addressed meaningfully, it must arise in a case in which the decision will turn on that question, and, recognizing this, the parties fully brief the issue. This is not that case. We conclude that it is not necessary to the disposition of this case to address the question raised by the Commissioner; accordingly, we decline the invitation to do so.

(*Brana* at 1443-44). The court's position is reflected in the MPEP: if an "assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility" (MPEP § 2107, II (A)(3); II (B)(1)). If it is well known to those skilled in the art that knockout mice are useful for studying gene function, then those skilled in the art would certainly regard such use as credible, specific and substantial. Nothing more is required to satisfy the statutory requirement. Applicant submits that, as in *Brana*, one skilled in the art would find the asserted use credible, substantial and specific.

In *Brana*, the court found a mouse with an implanted murine tumor to represent a specific disease. Applicant submits that the claimed mouse having with a null allele of the endogenous CX2 gene and demonstrating increased glucose tolerance, likewise represents a specific disease.

6. Additional Examiner Arguments

The Examiner cites Olsen for the proposition that a knockout mouse "may" not be capable of elucidating the function of the protein.

First, as pointed out by Doetschman, one clearly skilled in the art, (*Laboratory Animal Science* 49:137-143, 137 (1999)(copy attached), the phenotypes observed in mice do correlate to gene function:

> The conclusions will be that the knockout phenotypes do, in fact, provide accurate information concerning gene function, that we should let the unexpected phenotypes lead us to the specific cell, tissue, organ culture, and whole animal experiments that are relevant to the function of the genes in question, and that the absence of phenotype indicates that we have not discovered where or how to look for a phenotype.

(emphasis added)(copy previously provided).

Second, the Examiner's argument is based on conjecture, not fact. Third, even if true, whether the CX2 gene directly or indirectly causes these phenotypes is irrelevant – a drug targeting the gene or protein would have the same effect – directly or indirectly.

Fourth, Olsen is clearly unsupportive of the Examiner's position that the claimed knockout mice lack utility. Olsen states that "gene targeting is *useful* in delineating the contribution of a given gene product to phenotypic characteristics" even though "some gene knockouts lead to embryonic or perinatal lethality, and others lead to no apparent phenotype" (emphasis added). In fact, even with respect to GABA genes, Olsen concludes that "the use of mutant and knockout mice has aided understanding of the roles of GAD and GABAR in the

intact mammalian organism, with much promise for additional information to come" (Olsen at 91). Even with respect to mice having increased lethality, Olsen states: "[t]he γ2 and β3 subunit knockouts are associated with early postnatal lethality but have nonetheless provided considerable new information about their importance, include relevance to neurodevelopment, synaptogenesis, and possibly human disease. The β3 is a strong candidate for involvement in the epilepsy and other phenotypic attributes of Angelman syndrome, a human genetic disorder characterized by mental retardation, seizures, motor incoordination, and sleep disturbances. The γ2L knockout has allowed direct testing and negation of the selective subunit hypothesis for ethanol modulation of GABAR function. The δ subunit knockout appears to provide information about the function of GABAR in adult cerebellum, dentate gyrus of the hippocampal formation, and the thalamus. GAD_{65}, GABAR β3, and GABAR δ subunit knockouts all exhibit spontaneous seizures, but of different sorts, confirming suspicions that GABAR malfunction might produce epilepsy by more than one mechanism and *providing excellent animals models for investigation of the cause of the seizure phenotype*." (Olsen at 91-2).

Olsen goes further: "[i]n summary, transgenic and knockout mice have demonstrated that GABA plays a major role in brain development, control of palate formation, and epileptogenesis via multiple mechanisms." (Olsen at 92). It is untenable to cite Olsen as standing for the proposition that knockout mice do not have a well accepted or substantial use.

In the present case, the claimed CX2 null mouse in fact demonstrates phenotypes. Olsen would agree that such mice are clearly useful.

With regard to the number of mice used in the tests, Applicant's previously made statement is not contradictory. Applicant had stated that "all behavioral tests set forth in the Examples were performed using at least ten (10) homozygous mice and compared with at least ten (10) wild-type control mice (numbers not shown)." (In this case, according to DeltaBase records, nine F2N1 (-/-); ten F2N1 (+/+); two F2N0 (-/-); and two F2N0 (+/+) were analyzed. Under Deltagen's designations, N0 and N1 correspond to N1 and N2, respectively, as typically used in the art). For the glucose tolerance test, a non-behavioral test, Figure 4 indicates 6 mice were tested.

The Examiner argues that "C57BL/6 and various substrains of 129" are unusual on may standard behavioral paradigms, citing Crawley.

Applicant established baseline values for the 129, C57BL/6 and the F1 hybrid (129 x C57BL/6) mice for all behavioral tests conducted and reported in the specification. The data was then collected on intercrossed mice (F2 generation). While the Examiner argues that the credentials of Deltagen's pathologists is irrelevant, Applicant strongly disagrees. The issue of their credentials is highly relevant to the credibility of the reported phenotypes and their association with gene function, and in particular to the Examiner's questioning of the credibility of the asserted utility. Deltagen's staff was well aware of the effect of background on behavior and accounted for such effect using methods well established in the art, such as the establishment of baseline values, and use of backcrossed mice.

The Examiner argues that the phenotype is a reflection of the gene's interaction with a background.

This so-called hitchhiker allele effect is considered a rare phenomenon. According to Wolfer *et al.*,: "..the possibility exists that an apparent effect of a null mutation could be due to a flanking 129 gene. Generally, *the problem is disregarded* because it imposes control strategies deemed costly, and *because the statistically expected number of confounding flanking genes is relatively low*" (emphasis added) (2002, *TRENDS in Neuroscience*, 25:336-340; page 336)(copy attached).

The Examiner argues that the phenotype is dependent on what exogenous DNA is replaced with, and where the exogenous DNA is inserted.

The claims as amended reflect replacement of a functional portion of the CX2 gene with a LacZ-Neo cassette. It is known in the art that insertion of a Neo gene will normally result in ablation of function (see Hasty, previously submitted).

Scarff remarks that retention of a selectable marker "*can* lead" to "confounding phenoytpes." There is no evidence that the LacZ-Neo cassette is causing "confounding" phenotypes in the presently claimed invention. Moreover, it is unreasonable to expect the patentee to exclude this possibility. As argued above, Applicant has credibly set forth data showing that CX2 -/- mice possess phenotypes not observed in CX2 +/+ mice. One skilled in the art would accept that such findings indicate that the phenotypes are associated with the function of the CX2 gene.

7. Summary

In summary, Applicant submits that the claimed transgenic mouse, regardless of any disclosed phenotypes, has inherent and well-established utility in the study of the function of the gene, and thus satisfies the utility requirement of section 101. Moreover, Applicant believes that the transgenic mice are useful for studying CX2 gene function with respect to the cited phenotypes, for studying gene expression, and are therefore useful for a specific practical purpose that would be readily understood by and considered credible by one of ordinary skill in the art.

In light of the arguments set forth above, Applicant does not believe that the Examiner has properly made a *prima facie* showing that establishes that it is more likely than not that a person of ordinary skill in the art would not consider that any utility asserted by the Applicant to be specific and substantial. (*In re Brana*; MPEP § 2107).

Rejection under 35 U.S.C. § 112, first paragraph

The Examiner has rejected claims 29-32, 36, 38-40 and 42-46 under the first paragraph of 35 U.S.C. § 112 because one skilled in the art would allegedly not know how to use the claimed invention as a result of the alleged lack of either a specific or substantial asserted utility or a well-established utility for the reasons set forth in the utility rejection. Applicants respectfully traverse the rejection. For the reasons set forth above, it is Applicant's position that the claimed invention satisfies the utility requirement. Therefore, one skilled in the art would know how to use the invention.

The Examiner argues that the Applicant did not argue the enablement rejection "separately."

Applicant respectfully requests the Examiner to explain this comment. The Examiner had previously argued, under a combined section 101/112(1) rejection, that that invention lacked utility and therefore one would not know "how to use" the invention. No arguments under section 112(1) were made separately. Applicant responded to the Examiner's arguments with regard to section 101 and concluded that since the invention possessed patentable utility, one skilled in the art would know how to use the invention. It is Applicant's understanding that it has fully responded to the outstanding rejections.

Rejections under 35 U.S.C. § 132

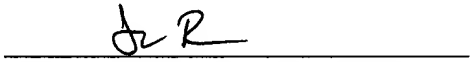
The specification had been amended to update cited application information. Previously cited U.S. Patent Application Ser. No. 08/971,310, filed November 17, 1997 (and now abandoned) was converted to 60/084,194, on which issued US patent no. 6,815,185 depends as a priority application filing. Applicant submits that the amendment does not recite new matter as the only document incorporated by reference is the disclosure of the originally cited 08/971,310 application. US patent no. 6,815,185 is only being cited as a publicly available document which contains the disclosure of the '310 application.

In view of the above amendments and remarks, Applicant respectfully requests reconsideration and a Notice of Allowance. If the Examiner believes a telephone conference would advance the prosecution of this application, the Examiner is invited to telephone the undersigned at the below-listed telephone number.

The Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. **502775**.

Respectfully submitted,

9-20-05
Date



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Techniques & Applications

Knockout mice: simple solutions to the problems of genetic background and flanking genes

David P. Wolfer, Wim E. Crusio and Hans-Peter Lipp

Inducing null mutations by means of homologous recombination provides a powerful technique to investigate gene function and has found wide application in many different fields. However, it was realized some time ago that the specific way in which such knockout mutants are generated can be confounding, making it impossible to separate the effects of the induced null mutation from those of alleles originating from the embryonic stem cell donor. In addition, effects from null mutations can be altered on different genetic backgrounds. Here we present some simple breeding strategies to test for flanking gene effects that are compatible with the recommendations of the Banbury Conference on Genetic Background in Mice and with common practices of creating and maintaining mouse knockout lines.

Recently, it has been realized that the experimental design of many studies in which genes were 'knocked-out' by means of homologous recombination are affected by a confounding factor: the flanking-gene problem [1,2]. Briefly, the problem is a consequence of the fact that the embryonic stem (ES) cells used in many such experiments are derived from substrains of 129 INBRED (see Glossary) mice [3]. A CHIMERA in which such ES cells populate the germ line will transmit not only the induced null mutation, but also the 129 genetic background, in their germ lines. Such males are usually mated to females from another inbred strain (generally C57BL/6), and the F1 hybrid offspring are mated with their siblings to produce the F2 generation. As a result, animals from this F2 generation will segregate not only for the induced null mutation and its wild-type ALLELE, but also for any other alleles at loci where the parental strains differ. When comparing null mutants with wild-type animals, most of these segregating genes will not pose any serious problems, as their distributions will be independent from the null mutation. This is different, however, for any genes linked to the targeted gene. Here, a so-called linkage disequilibrium will exist, because the null mutation will be flanked by two 129-derived alleles, and the wild-type locus flanked by two C57BL/6-derived alleles (Fig. 1). Therefore, the possibility exists that an apparent effect of a null mutation could be due to a flanking 129 gene. Generally, the problem is disregarded because it imposes control strategies deemed costly, and because the statistically expected number of confounding flanking genes is relatively low. However, active search for flanking-gene effects has indeed revealed candidate cases [4–6].

Glossary

Allele: one of two or more forms of a gene, reflecting intraspecies variance at a particular gene locus. An organism is homozygous for a gene if the alleles are identical, and heterozygous if they are different.
Chimera: when embryonic stem (ES) cells from a donor strain (e.g. 129) harboring targeted mutations are injected into blastocysts of a recipient strain (e.g. C57BL/6) and implanted into foster mothers, chimeras result. The chimeric animals that are born carry a conglomerate of cells from both strains. Some implanted ES cells differentiate into germ-line cells transmitting the genome (including mutation) of the donor strain, and chimeras can, thus, be mated to generate mutant mouse lines.
Co-isogenic strains: two inbred strains that carry different alleles of only one gene, and that are otherwise genetically identical [a]. Co-isogenic strains can arise through spontaneous mutation or through the artificial introduction of a mutation (e.g. a targeted null mutation or a transgene) into an inbred strain (e.g. by backcrossing chimeras to ES donor strain).
Congenic strains: strains obtained by repeatedly backcrossing mice heterozygous for a recognizable mutation (as evidenced by PCR or particular phenotypes) to an inbred strain. After ten generations, the contribution of genes from the donor strain that are not linked to the selected locus will be, on average, <0.1% [a].
Dominant; recessive: these terms indicate how phenotypes are inherited. A recessive phenotype is one that occurs only when an animal is homozygous for a particular allele. A phenotype is dominant if it is detectable when an animal is heterozygous for the allele. Single genes can be dominant for some phenotypes but recessive for others.
Genetic drift: in smaller populations, the proportion of animals heterozygous for a particular gene is reduced progressively even under conditions of chance mating, because germ cells transmit only one allele and the other allele is lost. In a population, the loss of alleles is stochastic and increases steadily. Thus, separate lines of mice bred from a common F2 cross between two inbred strains will drift apart genetically with increasing numbers of generations and will show systematic phenotypic differences unrelated to any targeted mutation.
Haplosufficient: the amount of gene-product generated by one functional allele is sufficient for normal gene function. On the phenotypic level this results in the mutation being recessive. The opposite situation is haploinsufficiency, where the amount of gene-product generated by one functional allele is insufficient for normal function and the phenotype related to the mutation is dominant [b].
Hybrid vigor: crossing two inbred mouse strains results in an F1 progeny that is isogenic, but heterozygous, for all alleles creating strain-specific deficiencies. Typically, such F1 mice are active, viable, able to out-perform both parental strains in many physiological and behavioral parameters and show minimal inter-individual differences.
Inbred: a strain of mice is considered 'inbred' if the mice have been brother–sister mated (sib-mated) for at least 20 generations [c]. These mice are genetically identical (isogenic) – that is, their genes are homozygous at all loci (with the exception that males and females differ for any genes on the Y chromosome).
Phenotype: any measurable characteristic of an organism. A phenotype is the result of interactions between genes and the environment.

References

a International Committee on Standardized Genetic Nomenclature for Mice (1994) Rules and guidelines for genetic nomenclature in mice. *Mouse Genome* 92, 7–32
b Crusio, W.E. (2002) 'My mouse has no phenotype'. *Genes Brain Behav.* 1, 71
c Green, E.L. (1966) *Biology of the Laboratory Mouse*, Dover Publications

Interactions with genetic background and environment

Another possible complication in gene-knockout experiments is the fact that the PHENOTYPE resulting from a null mutation can depend on the general genetic background of mouse strains used for this research. Thus, congenic strains carrying the same null mutation can sometimes show widely divergent phenotypes, depending on the genotype of the recipient strain [7,8]. Recent examples are the findings of Mineur *et al.* [9] and Ivanco and Greenough [10].

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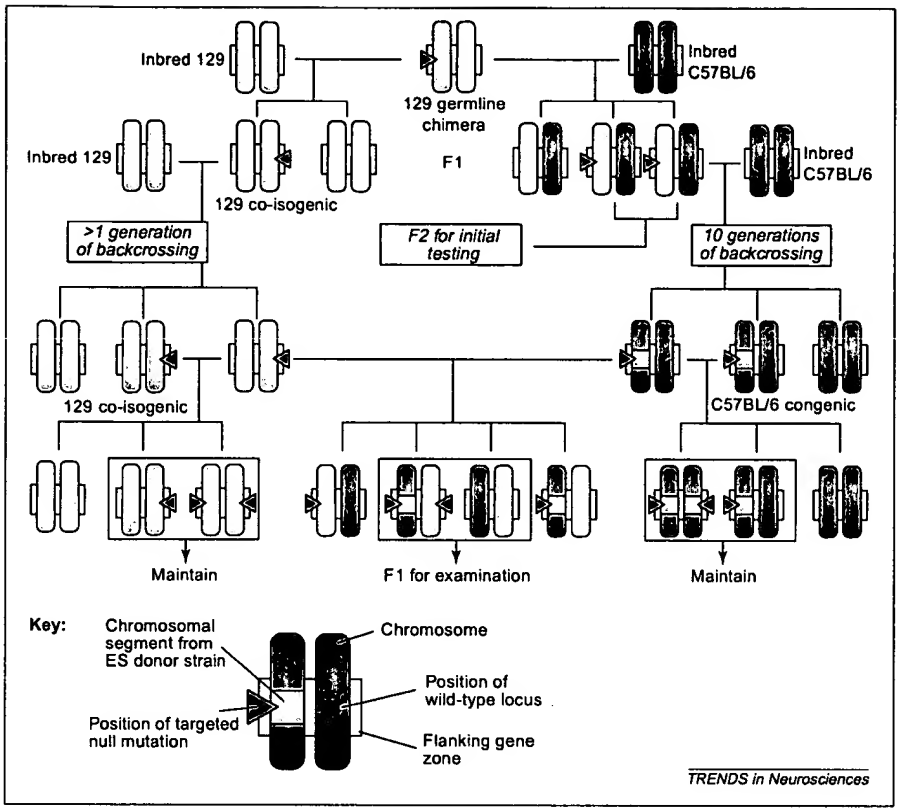

Fig. 1. Breeding strategies recommended by the Banbury Conference for maintenance of targeted mutations in congenic and co-isogenic lines, and for later production of genetically homogeneous F1-hybrid test samples [3]. The chromosome bearing the targeted locus is shown with background alleles originating from the embryonic stem (ES) cell donor strain (in this case, 129) in white, and those from C57BL/6 in blue (the scheme can also be applied to other strain combinations). During backcrossing, heterozygous mutants are repeatedly crossed with wild-type animals from an inbred strain until the strain is said to be congenic. Backcrossing of chimeras to the ES-cell donor strain establishes a co-isogenic line (this is advisable, as ES cells can accumulate genetic damage in cell culture). Because129 substrains often breed poorly, it can prove difficult to establish a co-isogenic line. As an alternative, a 129-congenic line can be established, starting from a population with mixed background and using predominantly inbred 129 males for backcrossing. However, this procedure is also likely to co-select some C57BL/6 genes that improve breeding success. Maintaining mutations by backcrossing to two different strains allows the repeated generation of F1-test samples by crossing the two lines. F1 hybrids are isogenic, even though they are heterozygous for loci showing allelic variation between the parental strains, which reduces their phenotypic variability remarkably. In fact, many behavioral traits show less variability in hybrid mice than in inbred strains, which is helpful in solving the standardization problem [11,12,22]. In addition, F1 (and also subsequent F2 and F3 generations obtained by intercrossing) benefit from hybrid vigor. Thus, F1 hybrids represent optimal samples for phenotypic testing [23]. By contrast, the use of test mice from a single congenic or co-isogenic line is discouraged because of potentially confounding effects of the genetic background (Fig. 2); a minimal requirement is that two congenic strains are tested. Maintaining a mutation in randomly bred animals from an initial F2 generation with segregating alleles from both strains (Fig. 2a) is a faulty strategy, particularly if wild-type and mutant animals are kept as separate lines. Owing to genetic drift, this will entail many phenotypic line differences unrelated to the targeted mutation.

They described opposite effects of a null mutation in the *Fmr1* gene, reporting an increase in the size of the hippocampal intra- and infrapyramidal mossy fiber terminal field when the mutation was on an FVB background, but a decrease when the mutation was on a C57BL/6J background. Finally, it should be realized that possible effects of a null mutation can also depend on the general laboratory environment [11] or on the particular behavioral testing method used [12,13]. However, we will concentrate on the genetic problems associated with targeted-gene experiments.

Box 1. Recommendations of the Banbury Conference on genetic background in mice [a]

General
- Reports of genetic experiments must include a detailed description of the genetic background.
- The genetic background should not be so complex as to preclude others from reproducing and expanding the reported experiments.
- The use of a common genetic background would facilitate the comparison of results across experiments and among laboratories.

Line maintenance
- Mutations should be maintained by backcrossing resulting in congenic or co-isogenic lines, preferably on both C57BL/6 and 129 backgrounds because these strains are used most widely.
- It is a faulty strategy to maintain separate mutant and control lines by continuous inbreeding of homozygous individuals starting from the original F2 generation, as this will entail genetic drift and, with it, phenotypic line differences unrelated to the targeted mutation.

Phenotypic characterization
- For the first characterization of a mutation, the F2 obtained by crossing the chimera to C57BL/6 and then intercrossing their heterozygous offspring offers a reasonable compromise between the demands of time and the rigorous control of genetic background.
- In the long term, it is recommended that mutants be analyzed on a defined hybrid F1 background, obtained by crossing two congenic or co-isogenic lines.

Reference

a Banbury Conference on Genetic Background in Mice (1997) Mutant mice and neuroscience: recommendations concerning genetic background. *Neuron* 19, 755–759

The flanking gene problem: inadequacies of the Banbury Conference recommendations

The Banbury Conference discussed breeding strategies for maintaining targeted mutations in mouse strains, and how to deal with the genetic background problem when breeding mouse samples for testing [3]. Thus, it was recommended that targeted mutations be maintained by backcrossing the initial F1 carriers with mice of two different inbred mouse strains, eventually producing CONGENIC and/or CO-ISOGENIC lines (Fig. 1, Box 1). This provides widely comparable mice that can be used for testing and, moreover, show HYBRID VIGOR. It also means that factors confounding phenotypic analysis arising from uncontrolled breeding schemes, such as GENETIC DRIFT, can be avoided. However, this strategy cannot solve the flanking gene problem. Even after backcrossing of a mutation for more than ten generations to a maintenance strain (usually C57BL/6),

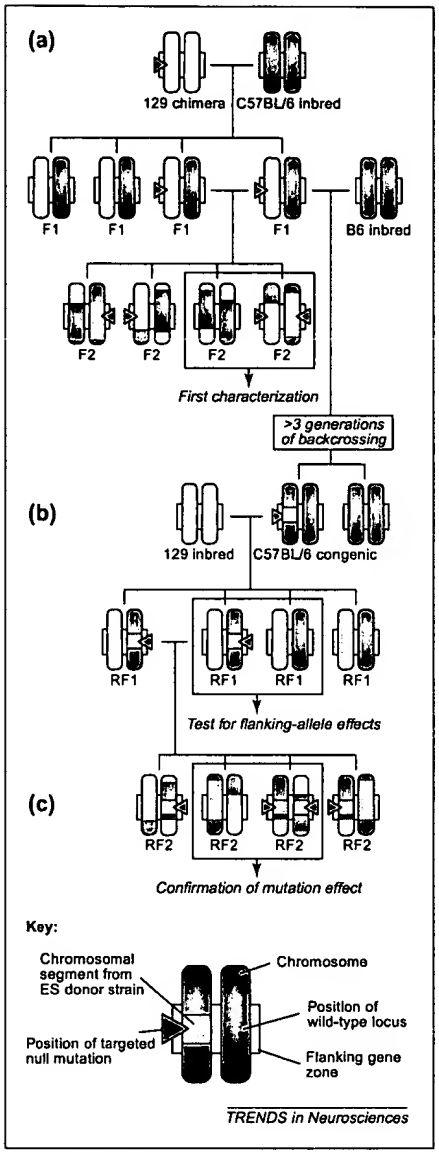

Fig. 2. A backcross–outcross strategy, to dissociate the effects of a targeted recessive mutation from those of flanking genes derived from the embryonic stem (ES) cell donor strain in a constitutive knockout model. The example uses the 129 strain as ES-cell donor, and assumes that C57BL/6 mice are used to generate test animals (although the scheme can also be applied to other strain combinations). Chromosomes or chromosomal segments originating from 129 mice are shown in white, those from C57BL/6 mice in blue. (a) A recessive mutation is generated by homologous recombination in ES-cells. The transmitting 129 chimera is mated to C57BL/6-inbred mice, to generate an F1 in which heterozygous offspring are intercrossed to produce an F2 generation. Homozygous F2 individuals are compared with wild-type littermates for a first phenotypic characterization [the appropriate statistical method to analyze the results would be a two-way ANOVA (analysis of variance), with genotype and litter as the main effects]. The genetic background of the F2 is largely heterogeneous, with equal contributions from the 129 and C57BL/6 genomes – except in the flanking gene zone. In this region, background alleles come from 129 in mutant animals, but come from C57BL/6 in littermate controls. Because 129 substrains often [24] (although not always [25,26]) show neuroanatomical and behavioral peculiarities [2,8,27–32], accumulation of 129 alleles in the flanking region of mutant animals could by itself produce a phenotype that is falsely attributed to the targeted mutation. Note that the C57BL/6 strain also harbors oddities, such as hearing impairments [33], enlarged ventricles [7] and poor performance in spatial-probe-trial tests [26]. Thus, using C57BL/6 ES cells does not eliminate the flanking gene problem. (b) Following the recommendations of the Banbury Conference (Fig. 1, Box 1), the mutation is maintained by backcrossing to C57BL/6 animals. This successively reduces the number of 129 alleles in the flanking region of chromosomes carrying the mutation, but does not eliminate them. In the course of backcrossing, some heterozygous individuals are outcrossed to inbred 129 mice, to generate a 'reverse F1' (RF1) generation, which differs from a 'true' F1 only in that individuals heterozygous for the mutation are all homozygous for 129 gene loci in the flanking region. The recessive mutation is, by definition, not phenotypically visible in this RF1. Thus, phenotypic differences in the RF1 that resemble the 'mutation' phenotype in the original F2 must be attributed to the flanking region, and interpreted as evidence for a contribution of homozygous recessive 129 flanking genes to the originally observed phenotype. Note that a large number of backcrosses reduces the sensitivity of this test, because 129 alleles are increasingly eliminated from the flanking region of chromosomes carrying the mutation. If the RF1 is generated too early, 129 alleles tend to predominate outside the flanking region as well as within it, which can also reduce sensitivity. The technique for creating speed congenics [14,15] is helpful to eliminate those alleles. (c) If no evidence for flanking-allele effects are found in the RF1, heterozygous mice are intercrossed to produce a 'reverse F2' (RF2), in which the mutation effect can be confirmed and dissociated from flanking-gene effects. In the RF2, the general genetic background resembles that of the original F2. In the chromosome region flanking the target locus, by contrast, most background loci will be homozygous for 129 alleles, independently of the genotype at the target locus. A contribution of flanking 129 alleles to the phenotype can be excluded if mutants in the RF2 show the same phenotypic difference from controls as in the original F2. Note that these models apply only if there is no functional interaction between targeted and flanking loci.

the resulting C57BL/6-congenic line will still harbor a part of the chromosome from the ES donor strain that contains a variable set of flanking genes depending on number of backcrosses. During backcrossing, there is continuous selective pressure for linked 129 genes, because only individuals heterozygous for the mutation are used to breed the next generation. Even after twelve generations, the chromosome segment containing genes from the ES-cell donor strain could contain as many as 300 genes, or 1% of the genome [2]. Likewise, mating only animals with genetic markers surrounding the targeted locus (speed congenics [14,15]) will not appreciably reduce the size of this chromosomal segment, unless the markers used are very close to the targeted locus and the researcher benefits from an improbably large dose of luck (i.e. obtaining cross-overs close to the targeted locus).

Available strategies to solve the flanking-gene problem

The flanking-gene problem might be solved by abandoning the classic strategy used to generate targeted mutations, as there are alternative models that are unaffected. For example, in so-called 'conditional knockout' models, wild-type controls can be substituted with mutants that have the mutation in the 'off'-state [16]. Alternatively, the normal phenotype of a constitutional null mutation could be rescued by re-introduction of the wild-type allele as a transgene [17]. Finally, one might use ES cells from other strains, such as C57BL/6 [18,19], to overcome some of the problems associated with the use of 129 stem cells, and backcross the chimeras to the donor strain C57BL/6. Such ES cells are now available*, and might permit a perfect (but costly) strategy for simultaneously producing co-isogenic lines from both 129 and C57BL/6 ES cells and testing their F1 hybrids. However, as already discussed, backcrossing alone (to C57BL/6, or to a 129-strain deemed more suitable for phenotypic testing) cannot eliminate the genetic background problem.

*To obtain C57BL/6 embryonic stem cells, contact K. Bürki (kbuerki@ltk.unizh.ch) at the Institute of Laboratory Animal Science at the University of Zürich.

Breeding strategies

Here we present some simple breeding strategies to deal with the flanking gene problem. The idea is to confirm the mutant phenotype by comparing littermates in which the alleles at flanking genes always come from the ES-cell donor strain, be this in wild-type or mutant animals. We present two such strategies. The first is a *post-hoc* strategy, for the majority of classic constitutional knockout mice, where a RECESSIVE (HAPLOSUFFICIENT) or incompletely DOMINANT null mutation is being conserved by backcrossing to one strain, usually C57BL/6. The second is a strategy useful for those who have created two congenic lines following the recommendations of the Banbury Conference. Two additional breeding schemes, based on other considerations, deal with less common situations.

The reverse F2 strategy

Commonly, the neurobehavioral effects of a recessive or incompletely dominant

targeted mutation are first characterized in an F2 generation (Fig. 2a), usually 12–18 months after generation of chimeras. If routine backcrossing for line maintenance to a C57BL/6 strain starts at the same time as the breeding of the first mice from chimeras, a partially congenic-C57BL/6 line can be created by making between four and six backcrosses (Fig. 2a). Because this C57BL/6-congenic line becomes available towards the end of the first characterization, often when a manuscript is at the draft stage, it can be conveniently used for ruling out flanking gene effects from crucial phenotypic changes before a manuscript goes to press.

C57BL/6-congenic mice are simply crossed with the ES-cell donor strain, which makes the flanking region of the resulting mutation-carrying animals homozygous for 129-derived alleles. A first comparison of these animals with wild-type F1 animals can indicate problems, if the mice heterozygous for the mutation then show similar phenotypic changes to those observed in the initial test sample (Fig. 2b). Further crossing results in a 'reverse' F2 generation, with a genetic background statistically similar to the F2 in which the phenotype was originally described (Fig. 2b,c), but which now has all flanking regions without the linkage disequilibrium (Fig. 2).

Testing crosses between two congenic lines

This strategy (Fig. 3) allows verification of a mutant phenotype previously characterized in an F1 that was produced by crossing two congenic lines. The phenotype is verified in a test population where heterozygous mutants and homozygous wild-type animals are otherwise genetically identical (i.e. they are heterozygous for genes where the 129 and C57BL/6 congenics differ and homozygous for genes where they carry the same alleles). This will be true for any chromosomal segment, including the region flanking the targeted gene (Fig. 3).

Complete dominance

To test for flanking genes suspected to mimic a dominant mutation effect in an initial F2 sample, simple crosses are sufficient. When the mutation had been backcrossed to the ES donor strain (e.g. 129), 129-congenics can be crossed with wild types from another strain (usually C57BL/6). This will give littermates of which 50% will be hybrid wild types, the other half being genetically identical except for the locus with the dominant mutation (as revealed by PCR). If the two groups still differ phenotypically, then the mutation must cause the difference. If they show equal phenotypes, dominant flanking alleles must be suspected.

Comparing two F2 populations

When an F2 generation is generated from a germ-line chimera and a C57BL/6 animal, one could simultaneously generate an F2 using wild-type 129 mice. Clearly, at most loci, both F2 generations will only differ because of chance variations due to segregation. The only systematic difference between the two groups will be the targeted gene, and any phenotypic difference can be ascribed to the null mutation, without confounding effects due to flanking genes [20]. However, even when large numbers of animals are used, it is evident that the statistical power of this design is not very great [21]. Further restrictions are that no littermate comparisons are possible, and that once the original chimera and any F1 animals derived from it are lost, this strategy becomes impossible.

Conclusions

The proposed breeding schemes require no specific technology, little resources and no more time than is already needed to comply with the recommendations of the Banbury Conference. Importantly, the 'reverse F2' strategy permits *post-hoc* testing for flanking gene effects, even when backcrossing is done only to the C57BL/6 strain (now a routine procedure in most laboratories). The only inconveniences are the replication of neurobehavioral testing some time after initial characterization, and the need to characterize phenotypically the inbred strains used to produce the knockouts. To us, this would seem good scientific practice rather than a nuisance and, compared to the risk of faulty conclusions due to confounding effects of flanking alleles, this seems a relatively small price to pay.

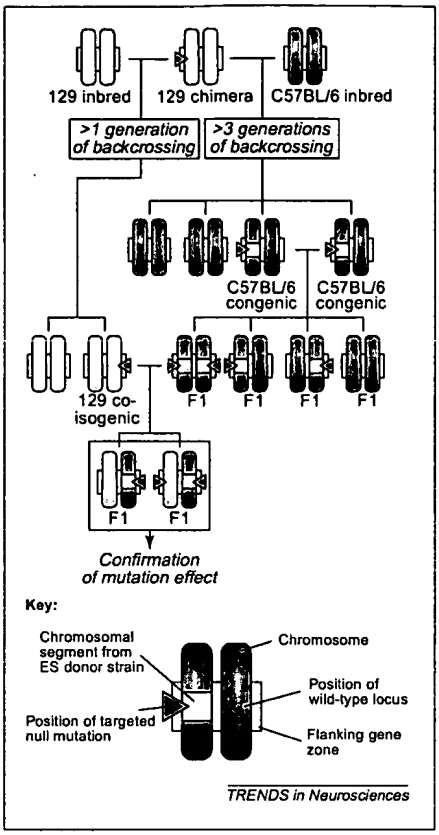

Fig. 3. Breeding strategy to dissociate the effects of a targeted recessive or incomplete dominant mutation from those of flanking genes, when the recommendations of the Banbury Conference have been followed (two C57BL/6- and 129-isogenic or 129-co-isogenic lines; Fig. 1, Box 1). Heterozygous individuals of the C57BL/6-congenic line are intercrossed to produce homozygous animals that can be selected and mated with heterozygous animals of the 129-co-isogenic line. This yields a test sample in which individuals are either homozygous or heterozygous for the targeted mutation. If the mutation is recessive, comparison of homozygous and heterozygous animals should reveal different phenotypes. With respect to genetic background, these animals are 129–C57BL/6 hybrids, except in the flanking region where genes will be homozygous for 129-derived alleles because of genetic linkage to the mutation. The degree of this predominance does not depend on genotype, however, because the genotype is determined by which chromosome is inherited from the 129-co-isogenic parent. Thus, flanking genes cannot contribute to phenotypic differences between homozygous and heterozygous animals in this population. Note that minor interpretation problems will arise if a mutant phenotype that was first described in a genetically heterogeneous F2 generation fails to reproduce fully in a test population with maximized hybrid vigor (which tends to reduce slightly any mutation effect). Thus, it will remain unclear whether this failure merely reflects the difference in hybrid vigor or whether it must be interpreted as evidence for flanking gene effects.

Acknowledgements

This work was supported by the Swiss National Science Foundation, the European Community (BIO4CT980297/BBW98.0125) and the NCCR grant 'Neural Plasticity and Repair'. We thank Robert T. Gerlai (Indianapolis, IN, USA) for critically reading an earlier version of the manuscript.

References

1 Büeler, H. *et al.* (1992) The neuronal cell surface protein PrP is not essential for normal development and behavior of the mouse. *Nature* 356, 577–582
2 Gerlai, R. (1996) Gene-targeting studies of mammalian behavior: is it the mutation or the background genotype? *Trends Neurosci.* 19, 177–181
3 Banbury Conference on Genetic Background in Mice (1997) Mutant mice and neuroscience: recommendations concerning genetic background. *Neuron* 19, 755–759
4 Kelly, M.A. *et al.* (1998) Locomotor activity in D2 dopamine receptor-deficient mice is determined by gene dosage, genetic background, and developmental adaptations. *J. Neurosci.* 18, 3470–3479
5 Bolivar, V.J. *et al.* (2001) Mapping of quantitative trait loci with knockout/congenic strains. *Genome Res.* 11, 1549–1552
6 Gerlai, R. (2001) Null mutant mice: an untapped source of quantitative trait loci. *Trends Neurosci.* 24, 470
7 Dahme, M. *et al.* (1997) Disruption of the mouse L1 gene leads to malformations of the nervous system. *Nat. Genet.* 17, 346–349
8 Magara, F. *et al.* (1999) Genetic background changes the pattern of forebrain commissure defects in transgenic mice underexpressing the β-amyloid-precursor protein. *Proc. Natl. Acad. Sci. U. S. A.* 96, 4656–4661
9 Mineur, Y.S. *et al.* (2002) Behavioral and neuroanatomical characterization of the *Fmr1* knockout mouse. *Hippocampus* 12, 39–46
10 Ivanco, T.L. and Greenough, W.T. (2002) Altered mossy fiber distributions in adult *Fmr1* (FVB) knockout mice. *Hippocampus* 12, 47–54
11 Crabbe, J.C. *et al.* (1999) Genetics of mouse behavior: interactions with laboratory environment. *Science* 284, 1670–1672
12 Van der Staay, F.J. and Steckler, T. (2002) The fallacy of behavioural phenotyping without standardization. *Genes Brain Behav.* 1, 9–13
13 Würbel, H. (2002) Behavioral phenotyping enhanced – beyond (environmental) standardization. *Genes Brain Behav.* 1, 3–8
14 Behringer, R. (1998) Supersonic congenics? *Nat. Genet.* 18, 108
15 Wakeland, E. *et al.* (1997) Speed congenics: a classic technique in the fast lane (relatively speaking). *Immunol. Today* 18, 472–477
16 Mansuy, I.M. *et al.* (1998) Inducible and reversible gene expression with the rtTA system for the study of memory. *Neuron* 21, 257–265
17 Kojima, N. *et al.* (1997) Rescuing impairment of long-term potentiation in *fyn*-deficient mice by introducing *fyn* transgene. *Proc. Natl. Acad. Sci. U. S. A.* 94, 4761–4765
18 Auerbach, W. *et al.* (2000) Establishment and chimera analysis of 129/SvEv- and C57BL/6-derived mouse embryonic stem cell lines. *BioTechniques* 29, 1024–1032
19 Schuster-Gossler, K. *et al.* (2001) Use of cotsogenic host blastocysts for efficient establishment of germline chimeras with C57BL/6J ES cell lines. *BioTechniques* 31, 1022–1026
20 Zimmer, A. (1996) Gene targeting and behaviour: a genetic problem requires a genetic solution. *Trends Neurosci.* 19, 470
21 Gerlai, R. (1996) Reply. *Trends Neurosci.* 19, 472
22 Wahlsten, D. (2001) Standardizing tests of mouse behavior: Reasons, recommendations, and reality. *Physiol. Behav.* 73, 695–704
23 Gass, P. *et al.* (1998) Deficits in memory tasks of mice with CREB mutations depend on gene dosage. *Learn. Mem.* 5, 274–288
24 Festing, M.F. *et al.* (1999) Revised nomenclature for strain 129 mice. *Mamm. Genome* 10, 836
25 Montkowski, A. *et al.* (1997) Behavioural performance in three substrains of mouse strain 129. *Brain Res.* 762, 12–18
26 Voikar, V. *et al.* (2001) Strain and gender differences in the behavior of mouse lines commonly used in transgenic studies. *Physiol. Behav.* 72, 271–281
27 Nguyen, P.V. *et al.* (2000) Strain-dependent differences in LTP and hippocampus-dependent memory in inbred mice. *Learn. Mem.* 7, 170–179
28 Balogh, S.A. *et al.* (1999) A behavioral and neuroanatomical assessment of an inbred substrain of 129 mice with behavioral comparisons to C57BL/6J mice. *Brain Res.* 836, 38–48
29 Simpson, E.M. *et al.* (1997) Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. *Nat. Genet.* 16, 19–27
30 Gerlai, R. (1998) A new continuous alternation task in T-maze detects hippocampal dysfunction in mice. A strain comparison and lesion study. *Behav. Brain Res.* 95, 91–101
31 Wolfer, D.P. *et al.* (1997) Assessing the effects of the 129/Sv genetic background on swimming navigation learning in transgenic mutants: a study using mice with a modified β-amyloid precursor protein gene. *Brain Res.* 771, 1–13
32 Wahlsten, D. *et al.* (2001) Behavioural testing of standard inbred and 5HT(1B) knockout mice: implications of absent corpus callosum. *Behav. Brain Res.* 125, 23–32
33 Carlson, S. and Willott, J.F. (1996) The behavioral salience of tones as indicated by prepulse inhibition of the startle response: relationship to hearing loss and central neural plasticity in C57BL/6J mice. *Hear. Res.* 99, 168–175

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Rodent models of prefrontal cortical function

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In this article, we consider whether studies in rats can provide useful information regarding the debate about the functions of the primate prefrontal cortex. At a superficial level, comparison of regional specializations within the prefrontal cortices of different species suggests functional correspondence. Unfortunately, the nature of functional specialization in primate prefrontal cortex is controversial, and data supporting the idea of homology between specific areas of rat and primate prefrontal cortex are weak. Nevertheless, we argue here that studies of the computational functions within the relatively undifferentiated prefrontal cortex of rats can shed light on processing in primate prefrontal cortex.

If any region of the cerebral cortex is unique to the evolution of primates, then the dorsolateral prefrontal cortex is likely to be it. The human dorsolateral prefrontal cortex is involved in many complex cognitive processes that have been described as 'executive' – that is, that they form a control system that coordinates cognitive sub-processes. For example, dorsolateral prefrontal cortex is thought to be involved in working memory and holding task-relevant information 'on-line' [1,2], supervisory attentional control [3], reasoning and decision-making [4] and the temporal organization of behaviour [5,6]. Despite the fact that their intellectual functioning seems to be spared, patients with damage to frontal cortex can suffer great personal and social difficulties [7]. Compromised prefrontal function is thought to underlie the myriad of complex cognitive deficits that accompany disorders such as Alzheimer's disease [8,9], schizophrenia [10,11] and Parkinson's disease [12,13].

Preuss pointed out that there was an absence of evidence, rather than evidence of an absence, for the rat prefrontal cortex including an area homologous to primate dorsolateral prefrontal cortex [14]. Preuss also noted that it was not necessary to postulate that the rat possessed a homologous area: there are considerable

  PII: S0166-2236(02)02164-1